selected method

A Referee Method for the Determination of Total Calcium in Serum[1]

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Introduction

The need for accuracy in clinical chemistry is now widely accepted. Copeland et al. (*1*) have stated that "There is presently increasing interest in the use of laboratory tests for the early detection of asymptomatic disease states, with the aim of preventing later overt manifestations of diseases. Long-term chronic disease also requires long-term follow-up. This requires that laboratory measurements for patient-care be comparable over a distance in time (i.e., the patient's lifetime) and (in the mobile population of the United States) over distance in geographic space." We believe that an analytical philosophy based on accuracy will accomplish these goals more directly than any other approach.

In all analytical systems, accuracy is more likely to be achieved when three criteria are fulfilled:

(*a*) There is agreement on a base system of measurement units.

(*b*) A well-characterized reference material has been produced.

(*c*) This material is used with a referee method of proven and demonstrated accuracy to realize in practice the property that is measured.

In applying these criteria specifically to the measurement of calcium in serum we note that:

(*a*) agreement on the atomic weight of calcium and on the definitions of the mole, kilogram, and liter (Système International d'Unités–SI units) has been established;

(*b*) calcium carbonate has been made available as a standard reference material (SRM 915) by the National Bureau of Standards;

(*c*) there is now available, as a result of this work, a referee method of known accuracy.

Since all the required components have now been produced it is possible, at least theoretically, to refer all measurements of serum calcium to a single common base of accuracy. Since it appears impossible to bring the results of multiple laboratories into coincidence (i.e., standardization) on any basis other than that of best achievable accuracy, a validated referee method is a first step toward this goal. However, improvement in the quality of results obtained every day in clinical laboratories is dependent on acceptance by the clinical laboratory field of a philosophy that demands capable and accurate routine methodology for analytical services. The sustained efforts of clinical chemists, professional societies, standards groups, manufacturers, and the appropriate government agencies will be needed to develop and validate methods and instruments by which user laboratories can achieve this target.

A full description of the rationale leading to the

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[1] This method evolved from a joint National Bureau of Standards and Clinical Laboratory Research Program to determine limits of accuracy in the clinical laboratory. J. P. Cali served as coordinator of the National Bureau of Standards team and Drs. Bowers and Young served as co-chairmen of the planning committee and the clinical laboratories team. Other members were A. Mather, R.N. Rand, and N. Radin. The evaluators of the method were the members of the clinical laboratories.

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development of referee methods has been included in the report of Cali (2) and a complete description of the development of the referee method for serum calcium has also been published (3). For those scientists interested in repeating the work, we suggest that they consult the complete report, because we only include the scope, limitations, and protocol of the method for serum calcium in the present paper.

The establishment of the accuracy of a measurement process from first principles alone is difficult and time-consuming. If alternative accurate methods exist against which the systematic biases or errors of the candidate method can be assessed, then the development time and effort can be much reduced. Fortunately for our work, a suitable baseline reference did exist, although it had not been applied to the analysis of physiological fluids. Calcium can be determined readily with an accuracy that is within a few tenths of one percent of the "true or absolute" value by a method based on isotope dilution–mass spectrometry. Because this technique was not generally available in clinical chemistry laboratories, it could not be used directly as the referee method; it has been described in detail in other publications (3,4) and will not be described here.

Scope and Necessary Conditions

The protocol is derived and adapted from the method of Pybus et al. (5). Subject to certain limitations, if the referee method is performed exactly as described, the concentration of calcium in serum can be determined to within ±2% of the true value. The method has only been evaluated over the concentration range of 1.5 to 3 mmol/liter (3–6 mEq/l or 6–12 mg/dl).

The referee method is not suitable for routine use in a clinical laboratory because of the volume of sample required and the time and precautions needed for analysis. The primary utility of the method is in:

(*a*) establishing the absolute value of calcium in control or pooled sera;

(*b*) establishing the validity of calcium methods used in various kits, reagent sets, and analytical processes;

(*c*) serving as a method against which the accuracy of the many field methods for calcium may be tested; and

(*d*) use by manufacturers of calcium reference materials, control sera, etc., to establish and maintain the quality of their products.

To attain the accuracy inherent in the referee method several important conditions must be met:

(1) Absolute adherence to the protocol is essential. Deviations, short-cuts, or adaptations are not permitted.

(2) All glassware such as pipets and volumetric flasks must meet NBS class A specifications.

(3) All reagents, including water, must meet specifications defined in the protocol.

This method has been evaluated with three different models of double-beam spectrometers (Model 153, Instrumentation Laboratories, Waltham, Mass., and Models 303 and 403, Perkin-Elmer Corp., Norwalk, Conn.). It is not known whether other instruments can provide the stability, precision, or linearity necessary to utilize the accuracy inherent in the referee method. It is certain that, whatever instrument is used, it must be in optimum working condition with great stability and linearity throughout the range of interest. All stages of the analytical procedure must be performed by well-trained analysts. All weighings, preparation, and removal of aliquots of the standard, blank, and unknown solutions must be performed with great care, to limit errors from these steps to less than 1%, if the overall accuracy of the method is to be achieved. The analyst must be prepared to commit as much as one week of his time to perform a series of analyses with this method.

This referee method is designed to provide accurate values for the concentration of calcium in an aqueous solution or serum. The method does not assure adequacy or integrity of sampling per se. However, if a representative sample is used, the method will yield an accurate value for the calcium in that specimen. It is therefore essential that a homogeneous sample is prepared for analysis.

Materials and Protocol

Reagent Specifications

1. Water (preferably distilled and de-ionized) should measure at least 10^6 ohms specific resistance at 25 °C. It should be available in large quantity for use as a diluent and for the final rinse operation on all glassware and apparatus coming in contact with the solutions involved. Only water that meets these specifications is to be used in these operations.

2. Calcium standard solutions should be prepared from $CaCO_3$ issued and certified by the National Bureau of Standards. Its identification number is SRM 915. This material should be dried for 4 h at 200 °C and cooled to room temperature in a desiccator before use.

3. Lanthanum oxide should be of high purity and known to contain less than 15 μg of calcium per gram (15 ppm).

4. Sodium, potassium, and strontium chlorides should be ACS Analytical Reagent Grade (AR) quality. These materials should be dried before use (see above).

5. Hydrochloric acid meeting ACS-AR specifications should be used.

Glassware

All glassware—10-ml volumetric pipet (to contain); 500-ml volumetric flasks—should meet NBS Class A specifications.

All glass or plastic surfaces coming into contact with reagents, water, diluent, or sample must have been previously cleaned as follows:

1. Use routine cleaning procedure (hot water with detergents, plus usual rinses).

2. Soak glassware overnight in HCl (1.0 mol/liter).

3. Rinse with several portions of distilled water (5–6 minimum).

4. Air dry (inverted) in a dust-free environment.

Preparation of Reagents

1. Stock blank solution (140 mmol of NaCl and 5.0 mmol of KCl per liter): To a clean 1-liter volumetric flask, add 8.18 g of NaCl and 373 mg of KCl. Dissolve in H_2O and fill to the neck. When at working temperature (ambient), dilute to the calibrated volume and mix by inverting the flask six times, let it stand for several minutes, and invert a further six times, and again twice immediately before reading.

2. Diluent solution (10 mmol of $LaCl_3$ and 50 mmol of HCl per liter): (Plan to make sufficient diluent for the work to be performed in one continuous series.) Transfer 1.63 g of La_2O_3 to a 1-liter flask and dissolve in 10 ml of H_2O and 6.7 ml of concentrated HCl. After the La_2O_3 is dissolved, dilute with water to the neck of the flask. When the solution has reached ambient temperature, dilute to calibrated volume and mix by inverting the flask as above. (*Note*: If an internal reference is to be used, add 30.6 mg of $SrCl_2 \cdot 6H_2O$ per liter.)

3. Standard stock solutions of calcium: Prepare a minimum of three concentrations at 2.00, 2.50, and 3.00 mmol of Ca per liter, with each to contain 140 mmol of NaCl and 5.0 mmol of KCl per liter. To each of three 1-liter volumetric flasks, add 8.18 g of NaCl and 373 mg of KCl. To the first flask (2.00 mmol of Ca per liter) add 200.2 mg of $CaCO_3$, to the second flask (2.50 mmol of Ca per liter) add 250.2 mg of $CaCO_3$, and to the third flask (3.00 mmol of Ca per liter) add 300.2 mg of $CaCO_3$. To each flask add a few milliliters of water and 1 ml of concentrated HCl. Make sure all the $CaCO_3$ is in solution before diluting with water to the neck. When at ambient temperature, dilute each flask to calibrated volume and mix by inverting the flask 30 times. Label all flasks appropriately.

Dilution Procedure

All solutions should be at a relatively constant ambient temperature.

All solutions except the diluent, but including the unknown samples, are diluted 50-fold, by using a 10-ml volumetric (to deliver) pipet and 500-ml volumetric flasks. Only *one* 10-ml volumetric pipet is to be used throughout, to reduce errors caused by differences in drainage times between the aqueous or dilute acid and sera solutions, and to eliminate errors caused by volumetric differences between pipets.

1. Transfer to a 500-ml volumetric flask approximately 450 ml of diluent stock solution. Add to the flask 10.00 ml of the blank stock solution by using the 10-ml pipet. After the pipet stops draining, gently blow out the residual liquid by using a rubber bulb. Rinse the pipet three times with diluent from the flask, each time returning the pipet contents to the flask by drainage and blowing.

2. Dilute to calibrated volume with diluent and mix thoroughly with 30 inversions. Set aside.

3. Aspirate into the pipet water contained in a clean beaker. Fill to slightly above the mark and discard.

4. To condition the pipet, fill to 1–2 mm above the mark with 2.00 mmol/liter Ca standard stock solution. Discard. Repeat this step twice.

5. To a 500-ml volumetric flask, transfer 10.00 ml of the 2.00 mmol/liter Ca standard stock solution by the technique described in steps 1, 2, and 3 above.

6. Repeat step 5 twice, but using the 2.50 and 3.00 mmol/liter Ca standard stock solutions. Condition the pipet each time (as in step 4), using the appropriate standard stock solutions.

7. After the blank and standard solutions have been diluted and the pipet rinsed with water, draw 2–3 ml of the first unknown solution or serum into the pipet. Place a finger over the end of the pipet and then withdraw from the unknown solution container. Tilt the pipet to a horizontal position and slowly rotate the pipet to wet thoroughly all internal surfaces. Allow a small amount of air to leak past the finger so that the rinse solution may come into contact (a small way above the mark) with the upper stem surface. Discard. Repeat the rinse and conditioning operation once more.

8. Fill the pipet to the mark with the unknown solution (or serum) and deliver into a clean 500-ml volumetric flask. (*Note*: The tip of the pipet should remain in the diluent to prevent the formation of foam by the serum.) Rinse the pipet three times with diluent solution, returning all rinses to the flask. Dilute the flask to calibrated volume with diluent solution and mix by inverting 30 times.

9. Rinse the pipet with water, condition with the next unknown solution (or serum), and repeat steps 7 and 8 as many times as there are unknowns to be analyzed. [*Note*: If the rinse solution does not completely wet the sides of the pipet, clean the pipet by rinsing it several times in a mixture of strong acids ($HCl:HNO_3$, 3:1 by vol). Then, repeat step 9.]

10. At the conclusion of the dilution procedures there should be:

(*a*) One 500-ml volumetric flask containing a 50-fold dilution of the blank stock solution. Label "B."

(*b*) Three 500-ml volumetric flasks each containing 50-fold dilutions of the calcium stock standard solutions. Label "2.00," "2.50," and "3.00" (mmol of Ca per liter).

(*c*) As many 500-ml volumetric flasks, each containing a 50-fold dilution of each of the unknown solutions (or sera) as there are to be analyzed. Label appropriately. (*Note*: For the sake of clarity in the following steps, it is assumed there is one unknown, labeled "X.")

Atomic Absorption Spectrometry (AAS) Measurement Procedure

It is assumed that the operator is fully familiar with the instrument to be used. It is not possible in

this method to give detailed instructions necessary to assure instrument stability, linearity, flame conditions, etc. Acetylene of highest purity only should be used. It should be withdrawn slowly so that acetone in the porous filler will not be drawn off. The air should be passed through a water trap and filter. Several of the requirements for optimum stability in reading calcium have been detailed previously (*5*). Instability caused by the following must be avoided:

1. main-line voltage fluctuation
2. noisy nebulizer
3. electronic instability
4. acetylene pressure below 414 kPa (60 lb./in.^{2})
5. moisture in the airline, air currents in the room
6. solvent residues in the premixing chamber or drainage siphon

In general, the full accuracy of the method cannot be attained unless the instrument is in optimum operating condition and meets all the specifications set forth by the manufacturer. Repeatability of readings of the same solution within ±1.0% (maximum) is a necessary condition. The use of integration, the strontium internal reference, and damping, where available will assist in the attainment of high precision.

1. Instrument and electrical adjustment: Prepare the atomic absorption spectrometer for operation according to instructions provided in the operator's manual. Place the calcium hollow-cathode lamp in the lamp-housing receptacle. Turn the power supply switch to "On." Select the optimum current for the lamp, and allow ample "warm-up" time for the lamp to become stable. Adjust the monochromator slit and set the wavelength selector to the calcium resonance line at 422.7 nm (4227 Å), or to the instrument's maximum energy setting between 420 and 425 nm.[5] Adjust the photomultiplier diode voltage to give optimum current output with minimum dark current.

2. Flame condition: Open the tank valves on the air and acetylene supplies. Adjust the secondary regulators as recommended by the manufacturer. Check the burner to make sure the premixing chamber and nebulizer are clean and free of any foreign obstructions. Insert an air/acetylene burner head [either a three-slot (Boling) or suitable single slot] on the burner. Light the burner and adjust the air and acetylene flow to rates recommended for the instrument. To stabilize the temperature of the burner head, aspirate water into the flame for at least 10 min before proceeding to the next step. (*Note*: A fuel-rich air–acetylene flame gives optimum sensitivity for the measurement of calcium; however, it may be difficult to obtain the precision specified in this method with a fuel-rich flame. Therefore, it is suggested that a stoichiometric or slightly fuel-rich flame be used to obtain the highest precision for calcium in serum.)

3. Determination of optimum absorption: Determine the stability and repeatability of the instrument as well as the calibration curve as follows:

(*a*) Adjust the instrument to zero absorbance while nebulizing water.

(*b*) Nebulize the solution of 2.00 mmol of Ca per liter, and measure the absorbance.

(*c*) If the absorbance is not 1.000, adjust the scale expansion of the readout system until the absorbance value is greater than 1.000. (*Note*: A scale expansion of approximately 5 is required for most instruments.)

(*d*) Readjust the instrument to zero absorbance with water.

(*e*) Nebulize the reagent blank, 2.00, 2.50, and 3.00 mmol of Ca per liter and record their absorbances. Nebulize water between each of the standard solutions and check the zero value.

(*f*) Repeat the sequence of blank and standards as outlined in 3*e* until a repeatability of readings for the same solution is within ±1.0%.

(*g*) Subtract the absorbance value for the reagent blank from the average value obtained for the standard solution.

(*h*) Plot on rectilinear graph paper the absorbance, corrected for reagent blank, as ordinate, vs. the concentration of the calcium standards, as abcissa, expressed in millimoles/liter. The calibration curve should appear as in Figure 1. (*Note*: If concentration values are determined directly from the instrument instead of the absorbance values, follow the procedure analogously, except use the blank to set the zero concentration.)

(*i*) If the calibration curve is not linear, prepare calcium standards of 2.25 and 2.75 mmol/liter, following the procedure given previously.

4. Absorbance measurement: Measure the absorbance of the unknown solution as follows:

(*a*) Repeat the calibration curve as outlined in step 3.

(*b*) Nebulize the unknown solution and then ne-

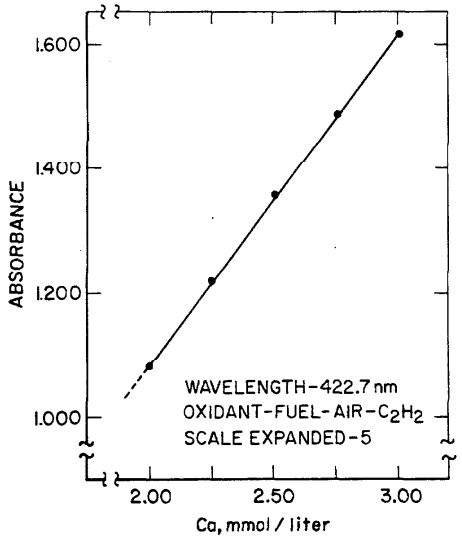

Fig. 1. Representative calibration curve for calcium

[5] Setting wavelength at the actual peak response (true emission line of the lamp) improves both the sensitivity of response and the stability of single-beam instruments.

bulize the two standard solutions that are closest to the value of the unknown. Record these absorbance values.

(*c*) Repeat this sequence of standards and a single unknown until 10 valid measurements have been obtained.

(*d*) Repeat steps 4*a* to 4*c* on additional unknowns and their associated standard solutions.

5. *Valid measurement*: To obtain a valid measurement, follow the sequence of standards and unknowns and record the data as follows:

		Ca, mmol/liter				
No.	*Sample*	*2.00*	*2.25*	*2.50*	*2.75*	*3.00*
1	Standards	1.084[a]	1.222	1.355	1.486	1.608
2	Unknown (1)			1.477		
3	Standards			1.354	1.480	
4	Unknown (2)			1.475		
5	Standards			1.351	1.488	
6	Unknown (3)			1.420		
				1.320	1.475	

[a] Typical absorbance values

In the preceding example, two valid measurements for the unknown in test 2 and 4 were obtained, because the difference between consecutive standards is less than 1%. However, the value obtained in test 6 is not valid because the difference between 1.351 and 1.320 is greater than 1%. That is to say,

$$1.355 - 1.354 = 0.001 \text{ or } <0.1\%$$
$$1.486 - 1.480 = 0.006 \text{ or } 0.4\%$$
$$1.354 - 1.351 = 0.003 \text{ or } <0.1\%$$
$$1.488 - 1.480 = 0.008 \text{ or } 0.5\%$$

6. *Calculations*: Calculate the sample concentration by an interpolation technique:

(a) Mathematical interpolation: Calculate the concentration in millimoles of Ca per liter for each valid measurement by using the following formula:

$$C = S_1 + \frac{(A_x - A_{s1})}{(A_{s2} - A_{s1})}(S_2 - S_1)$$

where C = sample concentration, mmol Ca/liter; S_1 = concentration of lower standard, mmol Ca/liter; S_2 = concentration of upper standard, mmol Ca/liter; A_x = absorbance of unknown; A_{s1} = absorbance of lower standard; and A_{s2} = absorbance of upper standard.

(b) Graphic interpolation: Plot the absorbance values for the upper and lower standards vs. concentration (mmol Ca/liter) on rectilinear graph paper for each set of data. Draw a straight line between data points and determine the concentration of the unknown from the curve.

(c) Computer interpolation: Use a least-squares plot of the upper and lower standards to compute the concentration of the unknown with a computer.

(d) Compile the concentrations obtained by method *a*, *b*, or *c* for the 10 valid measurements and determine the mean value. For the best precision, methods 6*a* or 6*c* are recommended.

Discussion

Five exercises, in which all the evaluators participated, were required before the present referee method was developed. The results obtained by this method show a slight negative bias (certainly less than 0.5%) when compared against the isotope dilution–mass spectrometric method. The earlier exercises of this study indicated that this bias is essentially negligible when compared with the larger random errors found between laboratories, even when the referee method is used and the instructions are followed exactly.

This referee method has been validated for the determination of calcium in serum and (or) solutions containing physiological quantities of sodium and potassium. When trained analysts follow the protocol exactly as described, and instrumental conditions are optimum, the referee method will produce results for the concentration of calcium accurate to within ±2% of the "true" or absolute value in the range from 1.5 to 3 mmol of calcium per liter of sample.

Inherent in the referee method are the conditions and findings of the candidate method of Pybus et al. (*5*) upon which this method was based. This is especially true for interferences and matrix effects. Significant departures from the method with regard to these factors may lead to substantial systematic errors. Both human and bovine pooled serum and aqueous solutions containing sodium and potassium at a physiological concentration may be analyzed with assurance by the referee method. The effect on accuracy of scaling down the sample size or of using a dilution of less than 10:500 has not been tested although some additional work (not reported here) indicates that scaling down and use of a sample aliquot in an identical dilution might be tolerated.

It is certain that a skilled analyst must perform the analyses if very accurate and precise results are to be obtained. Furthermore, the analyst must be thoroughly familiar with the spectrometer that he uses. The instrument must be in excellent operating condition, especially with respect to its stability and reproducibility.

Summary

The first referee method of analysis (a method of known accuracy) in clinical chemistry has been developed through the cooperation of scientists from the National Bureau of Standards and eight clinical chemistry laboratories. Subject to some limitations discussed in the text, calcium in serum can be determined by the referee method to within ±2% of the "true or absolute" value. The complete protocol, based on an atomic absorption spectrometric procedure, is described in detail.

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